INFECTION AND IMMUNITY, June 1987, p. 1431–1435
0019-9567/87/061431-05$02.00/0

Vol. 55, No. 6

Intestinal Mucus Gel and Secretory Antibody Are Barriers to *Campylobacter jejuni* Adherence to INT 407 Cells

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Received 2 September 1986/Accepted 7 March 1987

An in vitro mucus assay was developed to study the role of mucus gel and secretory immunoglobulin A (sIgA) in preventing attachment of *Campylobacter jejuni* to INT 407 cells. An overlay of rabbit small intestinal mucus was found to impede the attachment of *C. jejuni* to a monolayer of INT 407 cells. Mucus from rabbits previously colonized with *C. jejuni* was found to completely inhibit bacterial adherence to the underlying cells. Anti-*Campylobacter* sIgA was readily detected in mucus samples from previously exposed rabbits and was responsible for eliminating bacterial adherence to the INT 407 cells. This was shown by loss of inhibition after mucus absorption with *Campylobacter* cells. sIgA-containing mucus caused aggregation of the *C. jejuni* cells within the mucus layer of the assay system. Nonimmune mucus and sIgA alone were unable to cause bacterial aggregation, suggesting a cooperative role for mucus and sIgA. Antibodies responsible for adhesion inhibition were cross-reactive among several *Campylobacter* strains and were not directed solely against flagellar antigens.

The role of secretory immunoglobulin A (sIgA) in gastrointestinal immune responses remains unclear. sIgA binds to and agglutinates bacteria but is not thought to be bacteriocidal (10). Using an experimental cholera infection model, Freter (11) showed that *Vibrio cholerae* remained viable in the presence of specific sIgA but lost its ability to persist in the intestine. Rapid clearance of *Campylobacter jejuni* from the intestines of immune rabbits has also been shown (3). Several mechanisms may explain these events.

It is possible that sIgA exerts this protective effect by binding to the bacterial surface and causing aggregation of the bacteria (14). The presence of antibody-antigen complexes in the gut is known to stimulate the production and flow of mucus (17). Trapping of pathogens within the mucus and their subsequent expulsion have been observed for *Salmonella* sp. (23), *Vibrio* spp. (11), and the *Trichinella* parasite (1).

sIgA-coated bacteria may also be unable to move toward or bind to specific receptor sites necessary for persistence on the mucosal surface. Williams and Gibbons (27) noted that streptococci present in human saliva were coated with sIgA and that the presence of this sIgA significantly reduced the number of bacteria able to bind to human buccal cells. In addition, Magnusson and Stjernstrom (23) noted that sIgA-coated salmonella carried a reduced negative surface charge and that their affinity for the layer of mucus that covers the mucosal surface was increased.

Mucin glycoproteins are also known to bind avidly to bacterial surfaces (6, 8, 14). *Escherichia coli* and *C. jejuni* attach to nonimmune rabbit intestinal mucus films in vitro (18, 24). In addition, a mucus penetration assay was recently used to demonstrate retention to *Salmonella* lipopolysaccharide mutants within a layer of mouse intestinal mucus gel (D. C. Laux, J. J. Nivola, and P. S. Cohen, Abstr. Annu. Meet. Am. Soc. Microbiol. 1986, D-37, p. 72). Together, mucin and specific sIgA may even more effectively bind to and aggregate bacteria within the mucus layer. Pathogens subsequently trapped within the mucus may then be more readily removed by normal intestinal peristalsis. These events would be consistent with observations in immune rabbits challenged via the RITARD procedure (3; D. H. Burr, M. B. Caldwell, S. K. Kirtland, A. L. Bourgeois, R. Wistar, and R. I. Walker, Abstr. Annu. Meet. Am. Soc. Microbiol. 1986, B-237, p. 63).

The mucus layer that covers the intestinal epithelium is a substantial structure (25), consisting of a heterogeneous mixture of proteins and very large molecular weight glycoproteins called mucins (5). sIgA appears to bind to mucin glycoproteins through hydrogen or disulfide bonds (5, 10). In addition, the hinge region of the human IgA molecule is similar in both amino acid and carbohydrate properties to intestinal mucin glycoproteins (13). These structural similarities tend to make sIgA more soluble in mucus than other classes (or subclasses) of immunoglobulins. This property has led some to suggest that sIgA may form a monolayer of antibodies at the mucoluminal interface as a barrier to pathogens (13).

We report here the use of an in vitro mucus assay to assess the interactions of *C. jejuni* with tissue culture cells overlaid with mucus from normal rabbits and from rabbits previously challenged with *C. jejuni*. A cooperative role for campylobacter-specific sIgA and mucus in preventing bacterial adherence through aggregation of bacteria was suggested.

MATERIALS AND METHODS

Bacteria and growth conditions. A clinical isolate of *C. jejuni* (WR-6) was obtained from Walter Reed Army Medical Center, Washington D.C. Motile (M$^+$) and nonmotile (F$^-$) variants of *C. jejuni* strain 81116 were kindly provided by D. G. Newell (Center for Applied Microbiology, Salisbury, United Kingdom). Additional motile (BS) and nonmotile (B-NS) variants were obtained from Robert Black (Center for Vaccine Development, University of Maryland Medical School, Baltimore). *C. jejuni* strain HC, used in most experiments, was a blood culture isolate from a patient at the Bethesda Naval Hospital, Bethesda, Md. Additional strains were obtained from the Naval Medical Research Institute culture collection. The *E. coli* K-12 strains were supplied by Richard Wilson, *E. coli* Reference Center, Pennsylvania State University, University Park.

Radiolabeling of bacterial cultures. *C. jejuni* cells were

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UNCLASSIFIED

SECURITY CLASSIFICATION OF THIS PAGE

REPORT DOCUMENTATION PAGE

1a. REPORT SECURITY CLASSIFICATION Unclassified	1b. RESTRICTIVE MARKINGS
2a. SECURITY CLASSIFICATION AUTHORITY	3. DISTRIBUTION/AVAILABILITY OF REPORT Approved for public release; distribution is unlimited
2b. DECLASSIFICATION/DOWNGRADING SCHEDULE	
4. PERFORMING ORGANIZATION REPORT NUMBER(S) NMRI 87-61	5. MONITORING ORGANIZATION REPORT NUMBER(S)
6a. NAME OF PERFORMING ORGANIZATION Naval Medical Research 6b. OFFICE SYMBOL (If applicable)	7a. NAME OF MONITORING ORGANIZATION Naval Medical Command
6c. ADDRESS (City, State, and ZIP Code) Bethesda, Maryland 20814-5055	7b. ADDRESS (City, State, and ZIP Code) Department of the Navy Washington, D.C. 20372-5120
8a. NAME OF FUNDING/SPONSORING ORGANIZATION Naval Medical Research and Development Command 8b. OFFICE SYMBOL (If applicable)	9. PROCUREMENT INSTRUMENT IDENTIFICATION NUMBER
8c. ADDRESS (City, State, and ZIP Code) Bethesda, Maryland 20814-5055	10. SOURCE OF FUNDING NUMBERS PROGRAM ELEMENT NO. / PROJECT NO. / TASK NO. / WORK UNIT ACCESSION NO: N/A

11. TITLE (Include Security Classification)
Intestinal Mucus Gel and Secretory Antibody are Barriers to Campylobacter Jejuni Adherence to INT 407 Cells

12. PERSONAL AUTHOR(S)
McSweegan, E.; Burr, D.H.; Walker, R.I.

13a. TYPE OF REPORT	13b. TIME COVERED	14. DATE OF REPORT (Year, Month, Day)	15. PAGE COUNT
journal article	FROM _____ TO _____	1987	5

16. SUPPLEMENTARY NOTATION
in: Infection and Immunity v.55, n.6, June 1987, pp.1431-1435

17. COSATI CODES			18. SUBJECT TERMS (Continue on reverse if necessary and identify by block number)
FIELD	GROUP	SUB-GROUP	Bacterial Adhesion; Campylobacter infections; IgA, secretory; Intestinal mucosa; Rabbits

19. ABSTRACT (Continue on reverse if necessary and identify by block number)

20. DISTRIBUTION/AVAILABILITY OF ABSTRACT ☒ UNCLASSIFIED/UNLIMITED ☐ SAME AS RPT ☐ DTIC USERS	21. ABSTRACT SECURITY CLASSIFICATION Unclassified
22a. NAME OF RESPONSIBLE INDIVIDUAL Phyllis Blum, Information Services Division	22b. TELEPHONE (Include Area Code) 202-295-2188 / 22c. OFFICE SYMBOL ISD/ADMIN/NMRI

DD FORM 1473, 84 MAR 83 APR edition may be used until exhausted. All other editions are obsolete.

SECURITY CLASSIFICATION OF THIS PAGE
UNCLASSIFIED

radiolabeled with sodium [^{3}H] acetate as previously described (24).

Adhesion assay. A previously described adhesion assay was used to study campylobacter adherence (18). Briefly, INT 407 cells were seeded into 24-well polystyrene tissue culture plates and allowed to form monolayers overnight. The following day, the wells were washed twice with 0.5 ml of HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid)-Hanks (HH) buffer, pH 7.4. Radiolabeled *C. jejuni* cells were also washed and suspended in HH buffer. Immediately after the INT 407 cells were washed, 0.25 ml of approximately 10^9 CFU of ^{3}H-labeled campylobacter was added to each well. The tissue culture plates were then incubated at 37°C for 3 h. Subsequently, the wells were washed with HH buffer to remove nonadherent bacteria. Adherent bacteria were recovered by adding 0.5 ml of 0.5% sodium dodecyl sulfate (SDS) to each well and reincubating the plates for 2 h. Samples (0.25 ml) were then removed, and the level of radioactivity was determined.

Rabbit immunization. New Zealand White rabbits (Dutchlands Labs, Inc., Denver, Pa.) were fed approximately 10^{10} CFU of live *C. jejuni* HC by the method of Cray et al. (7). Rabbits were subsequently challenged with the RITARD procedure (4). Immunity was assessed by a rise in sIgA titer and rapid clearance of the bacteria (Burr et al., Abstr. Annu. Meet. Am. Soc. Microbiol. 1986).

Preparation of intestinal mucus. Rabbit small intestinal mucus was prepared by the method of Laux et al. (18). Mucus samples were collected from normal rabbits and from rabbits previously colonized with *C. jejuni* HC. All mucus preparations were dialyzed against HH buffer, pH 7.4, and adjusted to a protein concentration of 1.0 mg/ml. Protein was measured by the method of Lowry et al. (22). Some mucus samples were adsorbed with approximately 5×10^9 CFU of *C. jejuni* HC or *E. coli* K-12 for 1 h at 4°C. Bacteria were subsequently removed by centrifugation, and the adsorbed mucus was stored at 20°C.

Mucus inhibition assay. The INT 407 cell adhesion assay was used to study the ability of campylobacter strains to attach to INT 407 cells overlaid with a layer of intestinal mucus. Polystyrene wells containing monolayers of INT 407 cells were washed twice with 0.5 ml of HH buffer. Immediately after the wells were washed, 0.4 ml of rabbit small intestinal mucus (1.0 mg of protein per ml) was added to each well. Bovine serum albumin (BSA; 1.0 mg/ml) was added to control wells. *C. jejuni* cells were washed in HH buffer and resuspended to an optical density of 0.95 at 600 nm. After mucus or BSA had been added to the wells, 0.25 ml of *C. jejuni* was carefully added to each well. The bacterial samples were allowed to run down the sides of each well and across the upper surfaces of the mucus layers to reduce the effects of mixing. The tissue culture plates were then incubated at 37°C for 3 h. At the end of the incubation period, the wells were washed, and the number of INT 407 cell-adherent bacteria was determined as described above.

sIgA ELISA. Anti-*C. jejuni* IgA antibody was measured in rabbit intestinal mucus samples. Horseradish peroxidase conjugated to goat anti-rabbit sIgA was used to detect rabbit sIgA to preparations of *C. jejuni* HC flagella. Goat anti-rabbit sIgA was raised against rabbit colostral IgA, affinity purified (manuscript in preparation), and conjugated to horseradish peroxidase by Kirkegaard and Perry Laboratories, Gaithersburg, Md.). Rabbit intestinal mucus samples were also screened for anti-*C. jejuni* IgG antibody by using a horseradish peroxidase-labeled goat anti-rabbit IgG preparation. All samples were uniformly negative (enzyme-linked immunosorbent assay [ELISA] titer, <20) in this assay (data not shown). The peroxidase substrate used in the sIgA ELISA was ABTS (2,2'-azino-di[3-ethyl-benzthiazoline sulfonate]) and was measured at 414 nm.

TABLE 1. Adherence of *C. jejuni* strains to INT 407 cells overlaid with intestinal mucus

Strain	CFU, 10^4/well[a] ± SE			
	Immune mucus/cells[b]	Mucus/cells[b]	BSA/cells[b]	BSA/plastic[c]
HC	4.2 ± 0.1	12.0 ± 0.2	28.8 ± 0.1	5.5 ± 0.1
M[+] (81116)	11.1 ± 1.2	23.3 ± 1.3	50.2 ± 0.9	10.9 ± 0.1
F (81116)	3.1 ± 0.2	8.4 ± 0.3	10.0 ± 0.2	5.3 ± 0.2
BS	8.4 ± 0.7	24.6 ± 0.4	42.2 ± 2.5	11.8 ± 1.1
B-NS	7.1 ± 0.7	12.5 ± 2.2	20.2 ± 0.5	8.2 ± 0.8
E8	10.3 ± 0.3	14.3 ± 0.2	13.0 ± 0.8	7.8 ± 0.3
WR-6	21.5 ± 0.7	22.6 ± 3.0	76.4 ± 2.3	17.2 ± 1.5

[a] Mean CFU per well were determined from triplicate sets of wells.
[b] INT 407 cells were overlaid with 0.4 ml of intestinal mucus or BSA.
[c] Controls consisted of 0.4 ml of BSA added to empty polystyrene wells.

Flagellum preparation. *C. jejuni* HC flagellar antigen, used in adsorption and ELISA studies, was isolated by a modification of the method of Logan and Trust (20). The acid dissociation step was omitted to ensure the recovery of large-molecular-weight flagella with unaltered antigenic sites. Flagellum preparations were examined by high-pressure liquid chromatography. Samples were run on a Waters 840 (Waters Associates, Inc., Milford, Mass.) high-pressure liquid chromatography by using a WISP 710 injection system with an SW-125 Protein-Pak exclusion column. The running buffer was 10 mM Tris, pH 8.0, and the flow rate was 0.5 ml/min. Flagellum preparations were also examined by SDS-polyacrylamide gel electrophoresis. Samples were suspended in 20 mM Tris, pH 7.4, and solubilized in SDS buffer by boiling for 5 min. Electrophoresis was done by using 4% stacking gels and 9 to 16% gradient separation gels. Gels were run at a constant current of 10 mA per gel until the tracking dye entered the separating gel; the current was then increased to 15 mA per gel. Phosphorylase *b* (molecular weight, 92,000), BSA (66,200), ovalbumin (45,000), carbonic anhydrase (31,000), soybean trypsin inhibitor (21,500), and lysozyme (14,400) served as molecular weight standards. Protein bands were visualized by Coomassie blue staining.

Collection of intestinal secretions. The lavage technique of Elson et al. (9) was used to collect intestinal secretions for rabbits before and after infection with *C. jejuni* HC. Some lavage samples from rabbits infected with *C. jejuni* HC were adsorbed with *C. jejuni* HC flagella. Flagella (0.3 mg of protein) were ultracentrifuged at 100,000 × *g* for 1 h and suspended in 2.0 ml of lavage fluid from previously infected rabbits. The lavage samples were then incubated for 1 h at 4°C with occasional mixing. Lavage samples were then recentrifuged to remove the flagellum-antibody complexes and stored at −20°C.

Calculations. Results were expressed as the mean of triplicate sets of wells within a given experiment. All experiments were repeated at least three times. Student's *t* test was used to determine significance. *P* values exceeding 0.05 were considered not significant. Numbers of adherent bacteria were calculated from the formula: (cpm/well)/(cpm/ml × [ml/CFU]) = CFU/well.

RESULTS

Mucus inhibition assay. An overlay of intestinal mucus from normal rabbits was a greater impediment to campylo-

TABLE 2. Interaction of *C. jejuni* HC with mucus and lavage samples

INT-407 cell overlaid with:	CFU, 10^6/ well
Expt 1	
BSA	14.8 ± 6.8
Mucus	7.4 ± 0.1
Immune mucus	2.1 ± 0.2
HC-adsorbed immune mucus[a]	7.1 ± 0.6
Pre-HC lavage/mucus[b]	7.8 ± 0.6
Post-HC lavage/mucus[b]	2.6 ± 0.2
BSA/plastic control	3.1 ± 0.2
Expt 2	
BSA	12.3 ± 0.2
Mucus	6.8 ± 0.5
Immune mucus	2.6 ± 0.07
HC-adsorbed immune mucus	5.1 ± 0.5
K-12-adsorbed immune mucus	3.1 ± 0.2
BSA/plastic control	3.1 ± 0.07

[a] Immune mucus samples were adsorbed with approximately 5 ± 10^9 CFU of *C. jejuni* HC or *E. coli* K-12 for 1 h at 4°C.

[b] Rabbit intestinal lavage samples were obtained before and after exposure to *C. jejuni* HC. Lavage samples (0.1 ml) were mixed with 0.3 ml of normal rabbit mucus and used in the above mucus penetration assay.

bacter adherence to INT 407 cells that was an overlay of BSA (Table 1). The mucus overlay impeded the seven tested *C. jejuni* strains to a greater extent than did the BSA overlay. In addition, mucus obtained from rabbits previously colonized with *C. jejuni* HC was more effective in preventing bacterial adherence to the underlying INT 407 cells than was mucus from noncolonized, nonimmune rabbits. The immune mucus overlays reduced the adherence of strains HC, M^+, F^-, BS, and BN-S to background levels. Only strain WR-6 showed no significant difference in its interactions with immune and nonimmune mucus.

The ability of mucus for rabbits previously colonized with strain HC to effectively block cell adherence suggested that antibodies in the mucus could be associated with this phenomenon. To determine whether this was the case, mucus from rabbits fed strain HC was adsorbed with whole-cell preparations of *C. jejuni* HC before being used in the mucus inhibition assay. HC-adsorbed mucus from immune rabbits was no longer able to prevent the adherence of *C. jejuni* HC (Table 2). However, adsorption of HC-immune mucus with *E. coli* K-12 had no effect on the ability of immune mucus to impede *C. jejuni* HC (Table 2). *E. coli* K-12-adsorbed

TABLE 3. sIgA titers in rabbit intestinal mucus and lavage fluid measured by ELISA

Rabbit no.	Sample	sIgA titer[a]
82	Immune mucus	320
82	HC-adsorbed immune mucus	<20
Control	Normal mucus	<20
64	Immune mucus	320
64	HC-adsorbed immune mucus	<20
64	K-12-adsorbed immune mucus	320
71	Post-HC lavage	320
71	Flagellum-adsorbed post-HC lavage[b]	80

[a] sIgA titer measured against purified preparations of *C. jejuni* HC flagella.

[b] Lavage samples were adsorbed with a purified preparation of *C. jejuni* HC flagella.

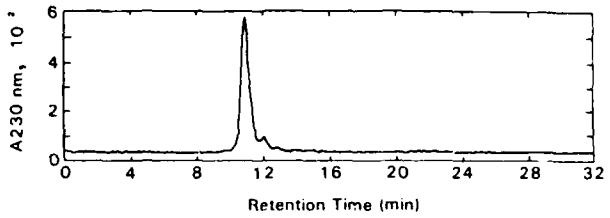

FIG. 1. High-pressure liquid chromatography elution profile of *C. jejuni* HC flagellum preparations. Flagellum samples were chromatographed on a Waters 840 high-pressure liquid chromatograph by using a WISP 710 injector and an SW-125 Protein-Pak exclusion column. The running buffer was 0.01 M Tris, pH 8.0, and the flow rate was 0.5 ml/min.

immune mucus was not significantly different from unadsorbed immune mucus in its ability to impede attachment ($P < 0.005$).

Lavage fluid, collected from rabbits before and after exposure to *C. jejuni* HC, was added to samples of normal mucus and used in the inhibition assay. Mixtures of normal mucus and post-HC lavage fluid were similar to immune mucus in their ability to block campylobacter adherence to the underlying INT 407 cells (Table 2). Pre-HC lavage fluid added to normal mucus was similar in effect to mucus from nonimmune rabbits.

sIgA quantitation. sIgA antibodies directed against *C. jejuni* HC were readily detected in both the mucus and lavage samples from previously exposed rabbits. sIgA titers were measured against *C. jejuni* HC flagella (Table 3). Adsorption of immune mucus with whole-cell preparations of *C. jejuni* HC significantly reduced the levels of sIgA, whereas adsorption with *E. coli* K-12 cells had no effect (Table 3).

Antibodies directed against antigens other than the flagella must contribute to retention of the bacteria within the mucus layer. Lavage fluid collected from HC-infected rabbits was adsorbed with HC flagella (Fig. 1 and 2) and used in subsequent inhibition assays. sIgA ELISA assays showed a fourfold reduction in sIgA against the flagella (Table 3), but this reduced antibody level failed to significantly increase the number of *C. jejuni* HC cells able to reach the INT 407 cells in the mucus inhibition assay (Table 4).

Campylobacter agglutination. Campylobacter in tissue culture wells containing immune mucus tended to aggregate into large clumps, whereas campylobacter in normal mucus or BSA did not (personal observation). Campylobacter cells

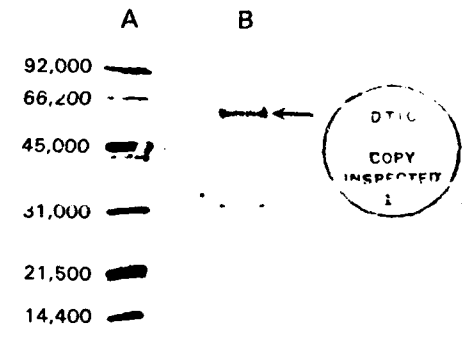

FIG. 2. SDS-polyacrylamide gel of flagellum preparation from *C. jejuni* HC. Protein standards (A) were routinely included in each gel. Molecular weights of standards are shown at left.

TABLE 4. Effect of flagellum adsorption on campylobacter interactions with rabbit lavage samples

INT-407 cells overlaid with:	CFU, 10^6/well
BSA	31.3 ± 2.5
Mucus	8.2 ± 0.2
Lavage/mucus (1:3)[a]	3.1 ± 0.1
Lavage/mucus (1:7)	4.2 ± 0.2
Flagellum-adsorbed lavage/mucus (1:3)	3.1 ± 0.1
Flagellum-adsorbed lavage/mucus (1:7)	3.9 ± 0.2
BSA/plastic control	4.5 ± 0.4

[a] Total volume of lavage fluid and mucus dilutions was 0.4 ml.

trapped within the immune mucus layers appeared to remain viable. Aggregates of campylobacter cells collected from some of the wells and streaked onto blood agar plates produced heavy lawns of bacterial growth.

In a subsequent experiment, microtiter agglutination assays were run with serial dilutions of immune mucus, normal mucus, and post-HC infection lavage fluid against suspensions of *C. jejuni* HC. Only immune mucus agglutinated campylobacter cells. The failure of *C. jejuni* HC to be agglutinated by sIgA-containing lavage fluid or normal mucus separately suggests a cooperative role for mucus glycoproteins and sIgA in the agglutinating and trapping of campylobacter cells.

DISCUSSION

C. jejuni is a motile, spiral-shaped organism that is ideally suited for traversing the mucus gel. It is chemotactically attracted to mucus (16), it is highly motile in the viscous environment of the mucus (19), and the flagellum does not bind to the mucus glycoproteins (24). Hazell and Lee (15) described mucus as "principally a medium permitting the passage of the bacterium to its colonization site." However, we have shown that its ability to adhere to mucus-covered cells is affected by the presence or absence of sIgA. The inhibition assay also demonstrated cross-reactivity among *C. jejuni* strains and the importance of mucosal antibodies directed against nonflagellar antigens. This assay may provide a convenient tool for dissecting immune responses to campylobacter infection, identifying important cross-reactive antigens, and determining the role of motility and chemotaxis in adherence.

In vitro assays are likely to be complicated and modified in vivo by factors that are not reproducible in currently used in vitro adhesion assays (12). Franek et al. (10) suggested that sIgA functions have been difficult to reproduce in vitro because of the loss of mucus normally associated with mucosal epithelial cells or because of the loss of important steric arrangement between sIgA and mucus.

We addressed some of the shortcomings of in vitro adhesion assays by using a system that employs a monolayer of INT 407 cells overlaid with a crude preparation of rabbit intestinal mucus gel. This system requires bacteria to interact with the mucus layer before being able to interact with the underlying cells. With this assay, we have demonstrated that the adherence of *C. jejuni* strains is significantly impeded by the mucus. This is consistent with a previous report showing that these same strains of campylobacter were able to bind to a film of mucus gel glycoproteins (24). In this report, binding to components of intestinal mucus reduced the number of *C. jejuni* cells able to reach and attach to the INT 407 cell monolayers.

Adherence to the underlying INT 407 cells was completely inhibited if the bacteria were presented with a layer of intestinal mucus that contained sIgA against *C. jejuni* HC. The importance of specific antibodies to campylobacter was shown when the mucus sIgA titers were reduced by preadsorption with *C. jejuni* HC whole cells. When this was done, mucus was no longer able to completely prevent campylobacter from attaching to INT 407 cells. However, it was important that mucus be present with the antibodies to be effective. Only when anti-*C. jejuni* HC lavage samples were added to nonimmune mucus was bacterial adherence to the underlying cells effectively eliminated.

Together, intestinal mucus and sIgA formed an effective barrier to bacterial attachment to the cells. In the presence of sIgA-containing immune mucus, *C. jejuni* HC cells were readily aggregated into large visible clumps within the mucus layer of the assay and in microtiter agglutination assays. Nonimmune mucus and lavage fluid from *C. jejuni* HC-colonized rabbits separately failed to agglutinate campylobacter cells. A similar agglutination reaction may be involved in the rapid, in vivo clearing of campylobacter from immune rabbits (3).

Antibody-containing mucus for *C. jejuni* HC-infected rabbits was effective in impeding several other strains of *C. jejuni*. This suggests that there is considerable cross-reactivity among the tested *C. jejuni* strains. Complete serotyping by the Penner and Lior systems (26) for these strains is not yet available, but Western blots and ELISA data both confirm the presence of cross-reactive antibodies from immune rabbits (manuscript in preparation).

During infection, antibodies are made to a variety of campylobacter outer membrane proteins (2, 26) and to the lipopolysaccharide (21). The flagellum has been identified as one of the immunodominant antigens of *C. jejuni* (26). Adsorption of immune lavage fluid with *C. jejuni* HC flagella reduced the sIgA titer against this antigen but failed to improve adherence by *C. jejuni* HC (Table 4). These data suggest that sIgA antibody is likely to be directed against additional antigens on the bacteria surface. This observation is supported by our data showing similar effects on immune mucus preparations on flagellated (M^+, BS) and aflagellated (F^-, B-NS) strains.

ACKNOWLEDGMENTS

This work was supported by the Naval Medical Research and Development Command, Research Work Unit no. 3M162770 A870AS309. E.M. is the recipient of a National Research Council Resident Research Associateship.

We are indebted to Paul S. Cohen of the Department of Microbiology, University of Rhode Island, for suggesting the development of the mucus inhibition assay used in this study. We thank Sarah Kirtland for performing the high-pressure liquid chromatography work, A. Louis Bourgeois for the sIgA ELISA results, and Richard Grays for providing the INT 407 cell monolayers.

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